SYNTHESIS AND BIOASSAY STUDIES OF CATIONIC PORPHYRINS FOR GENE TRANSFECTION DELIVERY

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To my beloved father, mother, brothers and dearest sister

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ABSTRACT

Porphyrins are stable aromatic tetrapyrrolic macromolecules found in many natural products such as hemin, chlorophylls and vitamin B_{12}. Interaction ability of cationic porphyrins with nucleic acid and their fluorescent properties for location identification in cellular domain have promoted their uses as potential gene vectors for gene therapy. In this study, basic cationic porphyrins bearing four positive charges were synthesized. Besides, amphiphilic porphyrins anchored with both hydrophobic and hydrophilic moieties were prepared to facilitate membrane penetration and to give a higher cellular uptake. Polyamidoamine (PAMAM)-porphyrin conjugate was also prepared to produce a complex with higher transfection level but with low toxicity. Adler-Longo condensation method was mainly used to synthesize these cationic porphyrin precursors. All cationic porphyrins were obtained in high yield. All of the compounds were characterized using ^{1}H-NMR, ^{13}C-NMR, ultraviolet (UV) and infrared (IR) spectroscopies. Cytotoxicity and cellular uptake of all cationic compounds were tested on Chinese Hamster Ovary (CHO) cells to evaluate their potential uses as gene carriers. Results revealed that all porphyrins show relatively low toxicity towards the cell even at high concentration (100 μM). The 5,10,15,20-tetrakis(*N*-methyl-4-pyridyl)porphyrin and two amphiphilic cationic porphyrins of 5-hexyl-10,15,20-tris(*N*-methyl-4-pyridyl) porphyrin and 5-propyl-10,15,20-tris(*N*-methyl-4-pyridyl)porphyrin which contain three positive charges on the periphery show the highest cellular uptake. It was also found that the amphiphilic *cis*-porphyrins of 5,10-dipropyl-15,20-bis(*N*-methyl-4-pyridyl)porphyrin and 5,10-dihexyl-15,20-bis(*N*-methyl-4-pyridyl)porphyrin exhibited higher cellular uptake compared to their *trans*-isomers, 5,15-dipropyl-10,20-bis(*N*-methyl-4-pyridyl)porphyrin and 5,15-dihexyl-10,20-bis(*N*-methyl-4-pyridyl)porphyrin.

ABSTRAK

Porfirina adalah makromolekul aromatik yang stabil dan boleh dijumpai dalam banyak sebatian semula jadi seperti hemin, klorofil dan vitamin B_{12}. Keupayaan porfirina kationik untuk berinteraksi dengan acid nukleik dan sifat pendarfluor porfirina yang dapat menentukan lokasi vektor di dalam sel menyebabkan ianya berpotensi untuk digunakan sebagai vektor gen bagi aplikasi terapi gen. Dalam kajian ini, porfirina kationik asas yang mengandungi empat cas positif telah disintesis. Selain itu, porfirina amfifilik yang bersifat hidrofobik dan hidrofilik telah disediakan untuk memudahkan penetrasi membran dan meningkatkan pengambilan bahan oleh sel. Konjugat poliamidoamina (PAMAM)-porfirina juga disediakan untuk menghasilkan komplek yang mempunyai tahap transfeksi yang lebih tinggi dengan ketoksikan yang rendah. Kaedah kondensasi Adler-Longo digunakan untuk mensintesis pelbagai bahan mula porfirina kationik. Semua porfirina kationik diperolehi dengan hasil yang tinggi. Semua sebatian telah dicirikan dengan menggunakan spektroskopi ^{1}H-RMN, ^{13}C-RMN, ultralembayung (UL) dan inframerah (IM). Kajian toksisiti dan pengambilan sel untuk semua sebatian kationik tersebut dilakukan dengan menggunakan sel Ovari Hamster Cina (CHO) untuk menilai potensinya sebagai pembawa gen. Keputusan kajian mendapati bahawa semua porfirina menunjukkan ketoksikan yang agak rendah walaupun pada kepekatan yang tinggi (100 μM). 5,10,15,20-Tetrakis(*N*-metil-4-piridil)porfirina dan dua porfirina kationik amfifilik, 5-heksil-10,15,20-tris(*N*-metil-4-piridil)porfirina dan 5-propil-10,15,20-tris(*N*-metil-4-piridil)porfirina yang mengandungi tiga cas positif menunjukkan pengambilan selular yang tinggi. Dapatan kajian juga menunjukkan *cis*-porfirina amfifilik, 5,10-dipropil-15,20-bis(*N*-metil-4-pyridil)porfirina dan 5,10-diheksil-15,20-bis(*N*-metil-4-piridil)porfirina menunjukkan pengambilan selular yang lebih tinggi berbanding dengan *trans*-isomer, 5,15-dipropil-10,20-bis(*N*-metil-4-piridil)porfirina and 5,15-diheksil-10,20-bis(*N*-metil-4-piridil)porfirina.

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LIST OF ABBREVIATIONS

ArCHO	-	Aromatic aldehyde
ANOVA	-	Analysis of variance
$BF_3.O(Et)_2$	-	Boron trifluoride etherate
br	-	Broad
^{13}C	-	Carbon-13
CHO	-	Chinese Hamster Ovary
COSY	-	H-H correlation spectroscopy
$CSCl_2$	-	Thiophosgene
d	-	Doublet
DCC	-	Dicyclohexylcarbodiimide
dd	-	Doublet of doublet
DDQ	-	2,3-Dichloro-5,6-dicyano-*p*-benzoquinone
DiTMPyP	-	5,10-Bis(*N*-methyl-4-pyridyl)-15,20-bis(4-pyridyl)porphyrin or 5,15-bis(*N*-methyl-4-pyridyl)-10,20-bis(4-pyridyl)porphyrin (**55**)
DMAP	-	4-(Dimethylamino)pyridine
DMSO	-	Dimethylsulfoxide
DNA	-	Deoxyribonucleic acid
DNases	-	Deoxyribonuclease
Et_3N	-	Triethylamine
FTIR	-	Fourier Transform Infrared Spectrometer
G_4	-	Generation four
h	-	Hour
^1H	-	Proton
IR	-	Infrared
J	-	Coupling constant
m-py	-	*meta*-Pyridyl
m	-	Multiplet

M	-	Molar
MeOH	-	Methanol
mg	-	Milligram
mL	-	Milliliter
mp	-	Melting point
MTT	-	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N_2	-	Nitrogen
NBS	-	*N*-bromosuccinimide
NCS	-	*N*-chlorosuccinimide
NHS	-	*N*-hydroxysuccinimide
nm	-	Nanometer
NMR	-	Nuclear Magnetic Resonance
o-py	-	*ortho*-Pyridyl
ODN	-	Oligodeoxyribonucleotide
OLED	-	Organic light emitting diode
PAMAM	-	Polyamidoamine
PDT	-	Photodynamic cancer therapy
PEI	-	Polyethylenimine
PLG	-	Poly(lactide-co-glycolide)
ppm	-	Part per million
PTSA	-	*p*-Toluenesulfonic acid
R_f	-	Retention factor
RNA	-	Ribonucleic acid
rt	-	Room temperature
SPSS	-	Statistical package for the social science version 16.0
STD	-	Standard deviation
TAPP	-	5,10,15,20-Tetrakis(4-acetamidophenyl)porphyrin **(38)**
TFA	-	Trifluoroacetic acid
THF	-	Tetrahydrofuran
TLC	-	Thin layer chromatography
TMPyCOPAMAM-		Cationic PAMAM G_4-porphyrin conjugate (**64**)
TMPyP4	-	*meso*-Tetrakis(*N*-methyl-4-pyridyl)porphyrin
TMPyHP2nd	-	5,10,15-Trihexyl-20-(*N*-methyl-4-pyridyl)porphyrin (**60**)

TMPyHP3rd	-	5,15-Dihexyl-10,20-bis(*N*-methyl-4-pyridyl)porphyrin (**61**)
TMPyHP4th	-	5,10-Dihexyl-15,20-bis(*N*-methyl-4-pyridyl)porphyrin (**62**)
TMPyHP5th	-	5-Hexyl-10,15,20-tris(*N*-methyl-4-pyridyl)porphyrin (**63**)
TMPyP	-	5,10,15,20-Tetrakis(*N*-methyl-4-pyridyl)porphyrin (**17**)
TMPyPP2nd	-	5,15-Dipropyl-10,20-bis(*N*-methyl-4-pyridyl)porphyrin (**57**)
TMPyPP3rd	-	5,10-Dipropyl-15,20-bis(*N*-methyl-4-pyridyl)porphyrin) (**58**)
TMPyPP4th	-	5-Propyl-10,15,20-tris(*N*-methyl-4-pyridyl)porphyrin (**59**)
TPP	-	Tetraphenylporphyrin
TriTMPyP	-	5,10,15-Tris(*N*-methyl-4-pyridyl)-20-(4-pyridyl)porphyrin (**56**)
UV	-	Ultraviolet
$Zn(OAc)_2$	-	Zinc acetate
q	-	Quartet
s	-	Singlet
t	-	Triplet
v/v	-	Volume per volume
µm	-	Micrometer
µM	-	Micromolar
µL	-	Microliter
δ	-	Chemical shift
λ	-	Lambda
%	-	Percent

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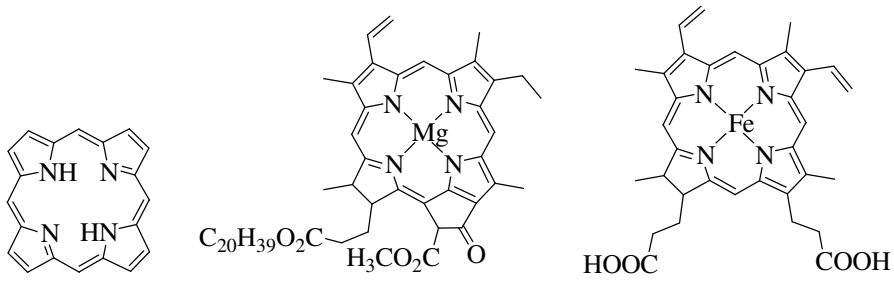
CHAPTER 1

INTRODUCTION

1.1 Background of the Study

Porphyrins **(1)** are important cofactor that can be found in massive amounts of natural products such as chlorophyll **(2)** and hemin **(3)** [1]. They are the central regulatory effectors in many biochemical processes. Over- or underproduction of porphyrins will result in significant health problems ranging from mental illnesses like schizophrenia, leukemia to physical symptoms such as, port-coloured urine.



(1) **(2)** **(3)**

Figure 1.1: Structure of porphyrin **(1)**, chlorophyll **(2)** and hemin **(3)**

Porphyrins can be accessed through laboratory synthesis and they are designed based on two porphyrins families; the *β*-substituted porphyrins that resemble naturally occurring porphyrins and the *meso*-substituted porphyrins. Porphyrins and their derivatives are usually synthesized based on condensation reactions between pyrroles and aldehyde derivatives since it represents a facile and straightforward synthetic method. Besides, different synthetic strategies also have been employed to obtain various porphyrin macrocycles. For example, MacDonald

reaction (2+2 acid-catalysed condensation) [2], Vilsmeier reaction, nucleophilic aromatic substitution reaction (S_NAr reaction) [3], electrophilic substitution reaction (S_EAr reaction) [4] and transition-metal catalysed reactions [5].

Porphyrins which consist of large aromatic macrocyles possess important chemical properties such as photochemical (energy and excitation transfer), redox (electron transfer, catalysis) [6] and coordination properties (metal and axial ligand binding) [7] which make these tetrapyrrole macrocyles play a crucial role in disparate areas like photodynamic cancer therapy [8-9], artificial photosynthesis [10], oxidation catalyst, sensors [11] and nanomaterial [12].

In addition, porphyrins and porphyrin-related macrocycles are found to have ability to bind to lysosomes, mitochondria and plasma membrane [8]. Cationic porphyrins have been reported for their ability to form strong electrostatic interaction with DNA [13] and used as nucleic acid transporting agents. For example, Kralova *et al*. reported that cationic porphyrin derivatives were efficient in transporting antisense oligodeoxyribonucleotides (ODNs) to primary leukemia cells [14]. Therefore, the electronic interaction with nucleic acids [15] and fluorescence properties has made porphyrins become promising delivery agents in gene therapy [16].

Gene therapy has been developed extensively as it provides a unique approach in treating both inherited and acquired disease. It involves the process of transferring genetic materials (DNA or RNA) into human cells to replace, correct or modify a mutated gene. To date, gene therapy has been shown as a successful tool to cure diseases such as, cystic fibrosis [17-18], severe combined immune deficiency (SCID) [19], haemophilia [20-21] and muscular dystrophies [22] as well as Parkinson disease [23]. Gene transfection process requires to surpass several barriers, starting from repulsion of negatively charged DNA by the negatively charged cell membrane, enzyme degradation of the DNA trafficks into endosome and finally internalization of the DNA through the nuclear pore into nucleus **(Figure 1.2)** [24]. Therefore, the gene delivery process has imposed formidable challenge to the development of gene therapy [14, 16].

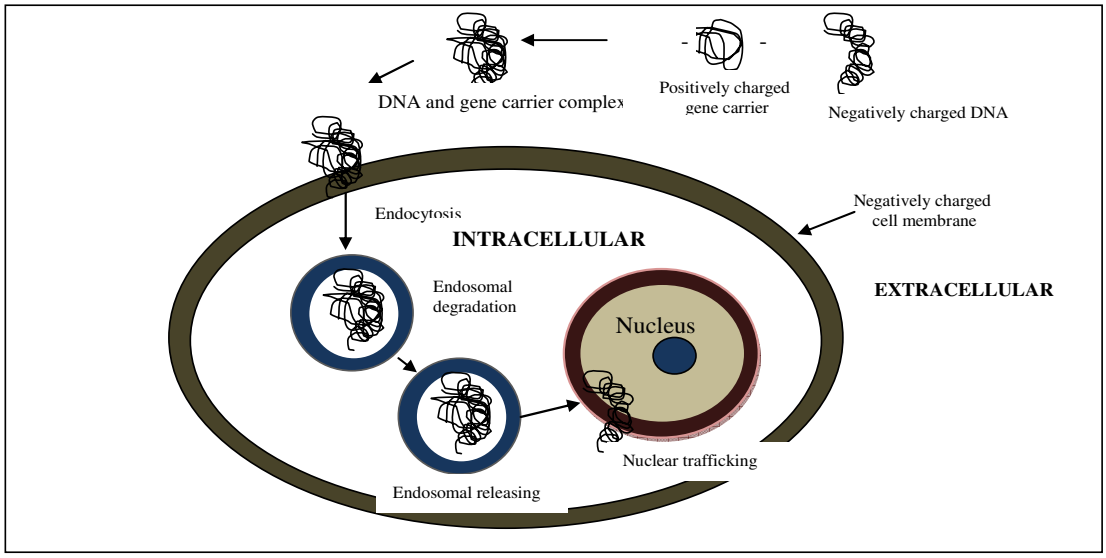


Figure 1.2 Gene therapy [24]

Retroviruses and adenoviruses have been used as viral vectors in gene therapy that may present higher gene transfection efficiency compared to their non-viral counterparts. However, viral vectors may trigger specific immune response and under certain circumstance could result in death. Their clinical trials indicated several concerns, such as mutagenesis [24], inflammatory properties [25] and high cost [22]. On the other hand, cationic polymers such as, polyethylenimine (PEI), poly-L-lysine, cationic dendrimers and hydrophobic polymers such as poly(lactide-co-glycolide) (PLG) or polyanhydrides that form polyplexes with DNA are one of the primary non-viral vectors systems that have been examined for the optimization of gene delivery [26]. These cationic polymers give protection to DNA from deoxyribonuclease (DNases) degradation as well as enhancing the intracellular uptake [27]. However, the clinical trials of these non-viral delivery vectors are limited by the issues of transfection efficiency, biocompatibility [28], toxicity [29], low targeting specificity, cellular uptake and tend to aggregate in the blood [24].

These drawbacks have stimulated the search for non-viral gene carriers with high biocompability and cellular uptake but give low aggregation and minimal safety concern. In this study, various cationic porphyrin derivatives were synthesized and their cytotoxicity and intracellular uptake were tested as an evaluation for their potential uses as gene delivering agent.

1.2 Problem Statement

The success of gene therapy largely relies on the ability of gene delivery vectors to deliver nucleic acid into the targeted cells with minimal toxicity [25, 30]. About 70% of current gene therapies are using the viral method as the main gene-therapeutic protocols [27], but it is plagued by some serious health issues as well as inflammatory response [25]. On the other hand, non-viral vectors present the issue of toxicity [29], low biocompatibility [28], low cellular uptake as well as tend to aggregate in blood [24].

Porphyrins are interesting alternative to viral vector-mediated gene delivery. As they are essential compounds in most natural products, such as haemoglobin, porphyrins probably have higher biocompatibility with human body cells and thereby, avoiding immune response as induced by viral vectors. Besides, both hydrophilic and hydrophobic substituents can be directly anchored on the porphyrins macrocycle to increase their cell membrane penetration, which in turn, increase their cellular uptake. Whereas, most non-viral vectors require the incorporation of natural lipid molecules for exogenous DNA transportation into the cell through a biomimetic mechanism [29]. As highly hydrophobic substituents may cause extensive self-aggregation in aqueous solution [15], a shorter length of hydrocarbon such as, butanal and heptanal were employed as hydrophobic substituents in this study.

Besides, porphyrin molecules can strongly fluorescent which favor the location identification of vectors in the intracellular domain [16]. On the contrary, a fluorescent probe is required to attach on most other non-viral vectors' surfaces for the delivery process tracking. The introduction of the fluorescent probe may interrupt the physical or chemical properties of the gene vectors, giving an adverse effect on the biocompatibility and transfection efficiency [28].

In addition, there are abundant of chemical substituents can be covalently bonded to porphyrin macrocylces for particular nucleic acid or cell-type application [31]. Polyamidoamine (PAMAM) dendrimers which have shown high level of

transfection through a “proton sponge” mechanism can be conjugated with porphyrin to form a complex that may enhance transfection level with minimal toxicity effect.

In this study, various cationic porphyrin derivatives with different number, location and distribution of lipophilic and hydrophilic ligands along the peripheral of the macrocycle were synthesized using condensation method and low cost reagents. To evaluate their potential as gene carriers with minimal toxicity effect, their cyctotoxicity and cellular uptake studies were conducted.

1.3 Objectives of the Study

The objectives of this study are:

1. To prepare and characterize basic cationic porphyrins.
2. To synthesis and characterize amphiphilic cationic porphyrin derivatives.
3. To synthesis and characterize cationic PAMAM-porphyrin conjugate.
4. To evaluate the cytotoxicity and cellular uptake of the cationic porphyrins as potential gene delivering reagents.

1.4 Scope of the Study

Condensation method was mainly employed in this study to synthesize diverse type of porphyrins. All cationic porphyrins were prepared using both methyl-*p*-toluenesulfonate and methyl iodide as the alkylation agents. Basic cationic porphyrin anchored with four cationic groups was synthesized. Besides, amphiphilic cationic porphyrins bearing different number of hydrophobic and hydrophilic substituents along the peripheral were also prepared in order to facilitate the penetration of gene carriers through the cell membrane. Polyamidoamine (PAMAM) dendrimer was also conjugated to porphyrins to produce a complex with improved cellular uptake and transfection level [32].

Cytotoxicity of the synthesized compounds towards the cells were conducted using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The assay was based on cleavage of yellow tetrazolium salt MTT to purple formazan by the metabolic active cells. In addition, cellular uptakes of the synthetic porphyrins were investigated in Chinese hamster ovary cells (CHO) and the qualitative results were obtained using inverted fluorescence microscope.

1.5 Significance of the Study

Porphyrins, as gene delivery agents may have a higher biocompability with human body cells as they are essential compounds in haemoglobin, thereby avoiding safety issue as triggered by viral vectors. Synthesis of amphiphilic cationic porphyrins give a better cell membrane permeability [33], which in turn producing a better cellular uptake. Self-aggregation is prevented when a shorter length of hydrocarbons were utilized as hydrophobic groups. Cationic PAMAM-porphyrin conjugate was also synthesized to enhance transfection level with minimal toxicity effect. In addition, the fluorescence properties of porphyrins contribute to the use of porphyrin as the marker candidate.

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